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Lead (Pb) contamination alters richness and diversity of the fungal, but not the bacterial community in pine forest soil

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The aim of the study was to gain a more detailed view of the changes in microbial community structure in boreal forest soil due to lead contamination. The Hälvälä shooting range in southern Finland is heavily polluted by lead. The humus soil microbial community was examined using DNA based methods. To examine the bacterial community a 400 bp section of the 16S rRNA gene was amplified, cloned, and sequenced. For fungi, the internal transcribed spacer (ITS) area was examined. The 917 bacterial sequences grouped into 398 operational taxonomic units (OTUs) at ≥ 97% similarity, while 649 fungal sequences grouped into 155 OTUs at ≥ 99% similarity. No effect of lead was found on bacterial richness or diversity, while fungal richness and diversity were significantly altered. Some OTUs assigned to Basidiomycota were much rarer in the lead contaminated areas while the genus Thelephora was enriched in the lead contaminated soil. This difference between bacterial and fungal responses to lead suggests a difference in the strategy to avoid the contaminant. The analyses performed here do not, however, allow for conclusions regarding mechanisms.

Introduction

Lead (Pb) is considered to be highly toxic to living organisms. In contrast to the wider-spreading lead pollution with an industrial origin, shooting range activities typically cause local contamination. In shooting range areas, lead concentrations can reach very high levels, and although visible effects on flora may not appear (Lin *et al*. 1995, Darling and Thomas 2003, Rantalainen *et al*. 2006), high contamination levels have been shown to cause serious threats to various vertebrates, including amphibians, birds and mammals (Stansley *et al*. 1997, Vyas *et al*. 2000, Lewis *et al*. 2001). However, due to the close contact between soil organisms and contaminants, soil biota can be expected to be the first and most directly influenced target, and supporting this, many species of soil fauna have been shown to be stressed by lead contamination (Hui *et al*. 2009, Salminen *et al*. 2002).

Microbial tolerance to lead has also been studied to some extent. The tolerance of bacteria to heavy metals, and also to other environmental stressors, is often related to plasmid-borne and/or transposon-borne traits (Sarand *et al*. 2000, Busenlehner *et al*. 2003). This suggests that the structure of bacterial communities does not need

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to change extensively in response to pollution if the microbes can evolve and equip themselves by receiving mobile genetic elements. However, our previous study showed that the composition of bacterial communities can also be altered to some extent by severe lead contamination (Hui *et al*. 2009). Fungi are commonly thought to be more tolerant to lead than bacteria, and fungal activity has in some cases been found to increase in heavy-metal contaminated soil (Rajapaksha *et al*. 2004). Although fungi in general tolerate higher concentrations of heavy metals than bacteria, the fungal community may still be affected by high heavy metal concentrations (Chander *et al*. 2001). For instance, lead was found to inhibit the growth of litter-decomposing fungi, although the level of inhibition seemed to vary considerably among fungal species (Hui *et al*. 2009). Similar findings were also reported by Baldrian (2003).

Previous studies have revealed that only about 0.3% of the microorganisms in soil are observable by means of traditional cultivation-based methods (Amann *et al*. 1995). Recently, microbial communities exposed to heavy metal pollution in forest soil have been described based on for example the analysis of fatty acid composition and 16S rRNA gene sequence variation using PLFA and T-RFLP, respectively (Åkerblom *et al*. 2007, Lazzaro *et al*. 2008). The results revealed specific microbial structural patterns and dominant bacterial groups under heavy metal contamination. The methods utilized, however, limited the resolution of the observations, since they reflect the community profiles, but do not report species. Many of the earlier studies have focused on bacteria, while fungi are largely ignored (Bååth *et al*. 2005, Hu *et al*. 2007). Moreover, to our knowledge, only a few studies have examined microbial communities under lead contamination in coniferous forest soil (Hui *et al*. 2009), whereas much attentions have been paid to other heavy metals, such as cadmium. The effects of different metals on microbes are, however, not always the same (Leyval *et al*. 1997).

The Hälvälä area is a heavily lead-contaminated site, but despite the soil being virtually covered with lead pellets, originating from long-term shotgun activity, the environment appears normal from a distance (Rantalainen *et al*. 2006). Earlier published measurements (Table 1; Hui *et al*. 2009) showed significant differences between the control and polluted areas regarding lead concentrations, but also regarding parameters such as pH and biomass. The water-soluble lead concentrations were, as expected, higher in the soil of the polluted area than in the control area, but the water-soluble lead represented only about 1% of the total lead (Hui *et al*. 2009). The biomass (including total, bacterial and fungal biomass) is significantly, although not dramatically, lower in the polluted area than the control area (Table 1; Rantalainen *et al*. 2006). The question arises: is the microbial diversity altered with the reduced biomass? The bacterial diversity seemed to be affected by lead contamination when determined using less than 150 16S rRNA gene sequences in Hälvälä soil (Hui *et al*. 2009). However, the low sequencing resolution provides insufficient evidence. In addition, the ectomycorrizal fungal communities under Pb contamination in Hälvälä soil was studied by means of "in-growth bag" and 454 pyrosequenc-

Table 1. Environmental factors in the soil in Hälvälä.

Area	Control	Polluted
Total Pb conc. (mg kg^{-1} DW)[a)]*	75.6 ± 49	13750 ± 10800
Total Pb conc. (mg kg^{-1} DW)[b)]*	106 ± 88	34540 ± 8650
Water soluble Pb conc. (mg kg^{-1} DW)[b)]*	3.2 ± 1.6	115 ± 26.6
pH[a)]*	3.25 ± 0.1	3.5 ± 0.26
Biomass total PLFA (μg g^{-1} OM of soil)[a)]*	1815 ± 437	1230 ± 436
Biomass bacteria PLFA (μg g^{-1} OM of soil)[a)]*	812 ± 219	580 ± 205
Biomass fungi PLFA (μg g^{-1} OM of soil)[a)]*	97.4 ± 34.4	47 ± 19
Tree age[a)]	21.6 ± 2.3	21.2 ± 2.1

[a)] Rantalainen *et al.* 2006, [b)] Hui *et al.* 2009; *found significant.

ing (Hui *et al*. 2011), but the total fungal diversity in soil remains unclear.

The aim of our study was to increase the understanding of the effects of lead pollution on soil microbial community structure (including bacteria and fungi) using DNA cloning and sequencing technology. So far, the effects of lead contamination on microbial communities have not been studied in forest soils in such detail. We hypothesized that, although the redundancy among the microbes seems to ensure that the soil system is fully functional as indicated by lack of influence on the flora, the species richness and diversity of bacterial and fungal communities would be altered by lead contamination.

Material and methods

Study site

The study site is a shot gun shooting range in a coniferous forest in Hälvälä, southern Finland (61º00´N 25º80´E). During the years 1964–1987, shooting was taking place in the old sector; since 1985, it has taken place in the new sector. The forest growing in the shooting sectors has not been managed in any way, and is therefore in natural condition. The soil type is well-stratified podsolic soil. Of the trees, Scots pine (*Pinus sylvestris*) dominates, while silver birch (*Betula pendula*) is found sporadically. The field layer is comprised of dwarf shrubs (*Vaccinium myrtillus*, *V. vitis-idaea*) and grasses (mainly *Deschampsia flexuosa*), while the ground layer consists of various mosses (mainly *Pleurozium schreheri* and *Dicranum* sp.) (Rantalainen *et al*. 2006).

Due to long-term use for shot gun shooting, the shooting range area is heavily contaminated by lead. We selected a lead polluted area, and an uncontaminated forest section nearby to represent a control area. The total Pb concentration (digestion in 8 M HNO_3) of the soil organic layer in the polluted area and control area (a nearby forest) were analyzed (Rantalainen *et al*. 2006, Hui *et al*. 2009) (Table 1). Lead contamination appears to have had a small but significant effect on the soil chemistry by increasing soil pH by 0.2 units (Rantalainen *et al*. 2006). Other area characteristics including ground vegetation were similar and the age of the dominant trees did not differ significantly (Rantalainen *et al*. 2006). The vegetation, terrain and visual appearance of the contaminated area were indistinguishable from the control area.

Sampling

The forest humus soils from the polluted area and the control area were sampled using a soil corer (diameter 3 cm, depth 10 cm, 30 soil cores per area) in September 2005. The soil was transported to the lab on ice in coolers and stored at 6 °C. The following day, plant roots, lead pellets, and the litter layer were removed, and the remaining humus was stored at –20 °C.

DNA extraction and PCR amplification

DNA was extracted from 0.5 g soil using Fast DNA® SPIN kit for soil (Obiogene Inc. Carlsbad, USA) according to the manufacturer's instruction. DNA was extracted from a total of 30 control area soil subsamples, and from 30 contaminated area subsamples. The DNA from ten subsamples was combined to form three final control area samples and three final contaminated area samples.

The whole bacterial 16S rRNA gene was amplified with primers pA and pH´ (Edwards *et al*. 1989). The 100 μl PCR reaction mixture contained 2 μM of each primer, 400 μM of each deoxynucleoside triphosphate, 1 mM of betaine, 2.5% of dimethyl sulfoxide, 1 μl of template DNA, 5 μl of F-516 10 × DyNAzyme buffer, 2 U of DyNAzyme II DNA polymerase (Finnzymes, Espoo, Finland) and 0.1 U of Pfu DNA polymerase (Fermentas, Vilnius, Lithunia). The thermal cycling program was as follows: initial denaturation at 94 °C for 5 min, followed by 24 amplification cycles of denaturation at 94 °C for 30 sec, annealing at 55 °C for 30 sec and elongation at 72 °C for 1 min, with a final elongation at 72 °C for 10 min.

For amplification of fungal ribosomal RNA genes, the fungal specific primer pair, FUN28f and ITS4: was used (White *et al*. 1990). The PCR reaction was performed in a 50 μl volume containing: 1 × PCR buffer (Finnzymes, Espoo,

Finland), 200 μM each dATP, dTTP, dGTP and dCTP, 0.3 μM primer, 1 U DNA polymerase (DyNAzyme II, Finnzymes, Espoo, Finland), 0.005 U Pfu polymerase (Fermentas, Vilnus, Lithuania) and 1 μl DNA template. The thermal cycling followed the program: initial denaturation at 94 °C for 5 min; 35 cycles of 94 °C for 1 min, 50 °C for 30 sec, 72 °C for 1 min 30 sec; followed by a final extension period of 10 min at 72 °C in a thermocycler (Tetrad, MJ Research, MA, USA).

DNA cloning and sequencing

The same DNA cloning method was applied to both bacterial and fungal DNA. The obtained PCR amplicons were purified with purification plates (Millipore, Massachusetts, USA, Partanen *et al*. 2010) using water suction (Ashcroft®, Berea, USA). To enable efficient ligation, A-nucleotide overhangs were inserted to the 5´ ends of the PCR products in a 50 μl reaction containing 5 μl of F-516 10 × DyNAzyme buffer, 250 μM of deoxynucleoside triphosphate and 1 U of DyNAzyme II DNA polymerase (Finnzymes, Espoo, Finland) at 72 °C for 1 hour. The products were purified with MicroSpin S-400 HR Columns (Amersham™ Biosciences, Little Chalfont Buckinghamshire, U.K.). The PCR products were cloned with Qiagen Cloning^{plus}-easy kit according to the manufacturer's instructions (Qiagen, Hilden, Germany) with the following variation: the ligation mixture contained 4 μl of insert DNA and 0.2 μl of vector solution. A 3 μl aliquot of the ligation mixture was used for the transformation. The rest of the cloning and bacterial sequencing procedure was carried out as described by Ala-Poikela *et al*. (2005) with following variations: inserts from clones were amplified using universal vector primers UP and RP (Partanen *et al*. 2010). Bacterial sequencing reactions were carried out with universal primer pD´ (Edwards *et al*. 1989). Fungal sequencing was done according to Hultman *et al*. (2010). All primers were obtained from Oligomer, Helsinki, Finland. Sequencing was done in the DNA Sequencing Laboratory, Viikki Biocenter, University of Helsinki by using ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit ver. 3.0 and analyzed on ABI Prism 3700 DNA sequencer (Applied Biosystems, Foster city, CA, US).

Sequence analysis

All sequences were analyzed and edited using the Staden Package (Staden *et al*. 2000), and inspected with Bellerophon's chimera detection program (Huber *et al*. 2004). Putative chimeric sequences were further checked with the Ribosomal Database II (RDP) chimera check program (Cole *et al*. 2003). In this study, bacterial sequencing was targeted at the variable regions V1 and V2 of the 16S rDNA which is about 400–500 bps (Neefs *et al*. 1993, Chakravorty *et al*. 2007). As suggested by Torsvik and Øvreås (2006), > 97% similarity was used for OTU (Operational Taxonomic Unit) assignment. Bacterial OTUs were analyzed by RDP Classifier (Cole *et al*. 2007) from the Ribosomal Database Project II and BLAST search at the National Center for Biotechnology Information website (McGinnis and Madden 2004).

Fungal sequences spanned the internal transcribed spacer (ITS) area of the ribosomal RNA gene cluster, containing the conserved 5.8 S rRNA gene and variable flanking ITS1 and ITS2 regions. The size of the fungal sequences ranges from 500–700 bps. Excessively short (< 300 bp) or poor-quality fungal sequences and the ones suspected of being chimeric based on ClustalW (Thompson *et al*. 1994) and database alignments were omitted. Sequences with > 99% similarity, as also used by Hultman *et al*. (2010), were assigned into OTUs in Gap4. Fungal OTUs were aligned with a BLAST search at the National Center for Biotechnology Information website (McGinnis and Madden 2004). Fungal taxonomic identification was made based on > 80% coverage and > 95% similarity of the reference database sequences.

Community attributes and statistical analyses

For the bacterial and fungal communities, the number of observed OTUs was used as an index of observed species richness, S_{obs}. Total species

richness was predicted using the Chao1 index $S_{Chao1} = S_{obs} + (n_1^2/2n_2)$, where n_1 is the number of OTUs observed once and n_2 the number of OTUs observed twice (Chao 1984, Hughes *et al.* 2001). Diversity of communities was estimated using a Shannon diversity index $H = -\sum P_i \ln P_i$ and Simpson's index $D = 1/\sum P_i^2$, where i goes through all observed OTUs and P_i is the proportion of the sequences of the ith OTU of all sequences. Evenness of communities was estimated using Shannon evenness $J = H/\ln S_{obs}$ and Simpson's evenness $E = D/S_{obs}$. To explore organismal coverage of bacterial and fungal dataset, species accumulation (rarefaction) curves were generated using EstimateS (ver. 8; Colwell 2006).

The statistical significance of the differences in the means of environmental factors, community attributes and frequency of individual taxa between the control and polluted areas were tested using a *t*-test.

Results

Bacteria

To characterize the bacterial communities, we cloned and sequenced a total of 1152 amplicons. After quality control, the final dataset contained 917 bacterial 16S rDNA sequences providing a sequencing depth of 153 ± 22 (mean ± 1SD) sequences per sample. The number of sequences was not significantly different between the control and polluted areas ($t = -1.14$, $p = 0.319$), indicating that the samples were accurately pooled. Obtained sequences were grouped into 398 OTUs with > 97% similarity (a representative sequence for each bacterial OTU has been submitted to NCBI Genebank under accession numbers HM069499–HM069896).

Species richness (number of observed OTUs, S_{obs}), diversity (Simpson's D and Shannon's H) and evenness of the bacterial community were not significantly different between the control and polluted areas (Table 2), indicating that there is no diversity differences between the areas with and without lead contaminations. The extrapolated species richness (S_{Chao1}) was not affected by lead pollution. However, the extent with which the extrapolated species richness exceeded the observed number of OTUs, suggests that, due to the shallow sequencing, the total bacterial diversity in both the control and the contaminated areas was inadequately covered (Table 2). A rarefaction analysis (Fig. 1) showed that the number of observed OTUs reached a plateau in neither the control nor the polluted area samples. Although the analysis does not report the total diversity, it does provide data on the identity of at least the major bacterial species and genera present in the samples.

In the analysis of the bacterial taxonomy, RDP and BLAST assigned 92% of the bacterial sequences into specific phylotypes (93% from the control and 91% from the polluted areas). The structure of the bacterial community is illustrated at five taxonomic levels (from genus to phylum)

Table 2. Number of observed OTUs (S_{obs}), extrapolated total number of OTUs (S_{Chao1}), and OTU diversity and evenness (exemplified by both Shannon and Simpson indices) for fungal and bacterial communities living in control and polluted forest areas ($n = 3$), and a *t*-test of the statistical significance of the difference between the area means.

	Fungi						Bacteria					
	Control area		Polluted area				Control area		Polluted area			
	Mean	SD	Mean	SD	*t*	*p*	Mean	SD	Mean	SD	*t*	*p*
S_{obs}	30	6.1	38	1.5	2.3	0.083	97	15.7	101	11.5	0.39	0.720
S_{Chao1}	52	2.6	42	3.6	3.83	0.019	361	44.4	431	72.5	1.43	0.225
Shannon diversity	2.74	0.33	3.29	0.12	2.72	0.053	4.31	0.17	4.27	0.16	0.3	0.782
Shannon evenness	0.81	0.08	0.91	0.02	2.06	0.108	0.94	0.01	0.93	0.02	1.77	0.152
Simpson's diversity	10.2	3.4	19.7	3.5	3.4	0.027	49.7	8.4	44.1	12.8	0.63	0.564
Simpson's evenness	0.34	0.12	0.51	0.07	2.09	0.105	0.51	0.07	0.43	0.10	1.13	0.321

in the pie diagrams for both areas (Fig. 2). On a phylum level, Proteobacteria-related OTUs comprised approximately 41% of the bacterial population in both control and polluted areas, and no statistically significant differences were observed between the areas in the frequency of the major lower taxa within this phylum (α-proteobacteria and Rhizobiales) (Fig. 2). The frequency of OTUs affiliated with Acidobacteria (34%) and Actinobacteria (10.5%), or the lower taxonomic levels within these phyla, were not affected by lead contamination either (Fig. 2). The frequency of Planctomycetes was twice as high in the control compared with that in the polluted area (Fig. 2), but the difference was not statistically significant due to the low frequency of these OTUs. No major bacterial community structure variation was noted when comparing the control and polluted areas, but lead may still affect minor bacterial populations. Some OTUs were found only in control or polluted areas. However, the low number of sequences did not allow for definite conclusions to be drawn regarding the effect of lead contamination.

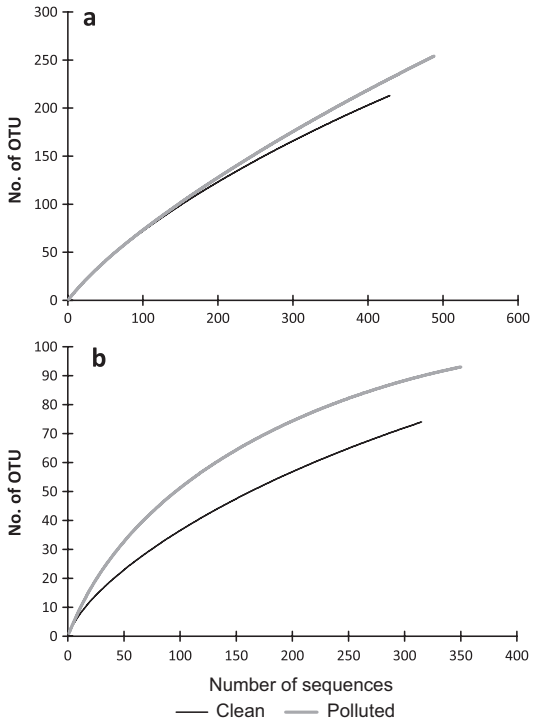

Fig. 1. Rarefaction curves for (**a**) bacteria and (**b**) fungi in control and lead contaminated areas.

Fungi

As 119 sequences were removed by fungal sequence quality control, 649 fungal ITS rDNA sequences were obtained, giving a sequencing depth of 108 ± 19 (mean ± 1 SD) sequences per sample. The number of sequences was not significantly different between samples from control and polluted areas (t = –0.38, p = 0.725), suggesting that each sample had been pooled in a manner that generated comparable samples. Of the 649 fungal sequences, a total of 155 OTUs were detected with > 99% similarity (a representative sequence for each fungal OTU has been submitted to NCBI Genebank under accession numbers HM069344–HM069498).

Significantly higher fungal richness (number of observed OTUs, S_{obs}) and diversity (Simpson's D and Shannon's H) were observed in the polluted than control area (Table 2). Both evenness indices were higher in the polluted than control area, but in neither case was the difference statistically significant (Table 2). The extrapolated species richness (S_{Chao1}) also revealed a difference between the areas, but in contrast to the observed richness, the predicted richness was significantly lower in polluted than control area (Table 2). Fungal diversity structure, differences in evenness and unsaturated sampling may explain this result. As in the case of bacteria, a rarefaction analysis (Fig. 1) revealed that the number of observed OTUs of neither the control nor the polluted samples reached the plateau, thus pointing to an unsaturated coverage of the total fungal diversity. This low coverage apparently lies behind the observation that the observed and predicted species richness are in apparent conflict with each other. In the polluted area, the number of observed OTUs (38) comes close to the total number (42) predicted by the Chao1 index, which indicates a relatively good coverage. In the control area, the coverage is poorer — 30 observed OTUs *vs*. 52 predicted by the Chao1 index.

Blast assigned 91.4% and 82.0% of total fungal OTUs to known phyla in control and polluted areas, respectively, indicating that there were more unclassified fungal sequences in the

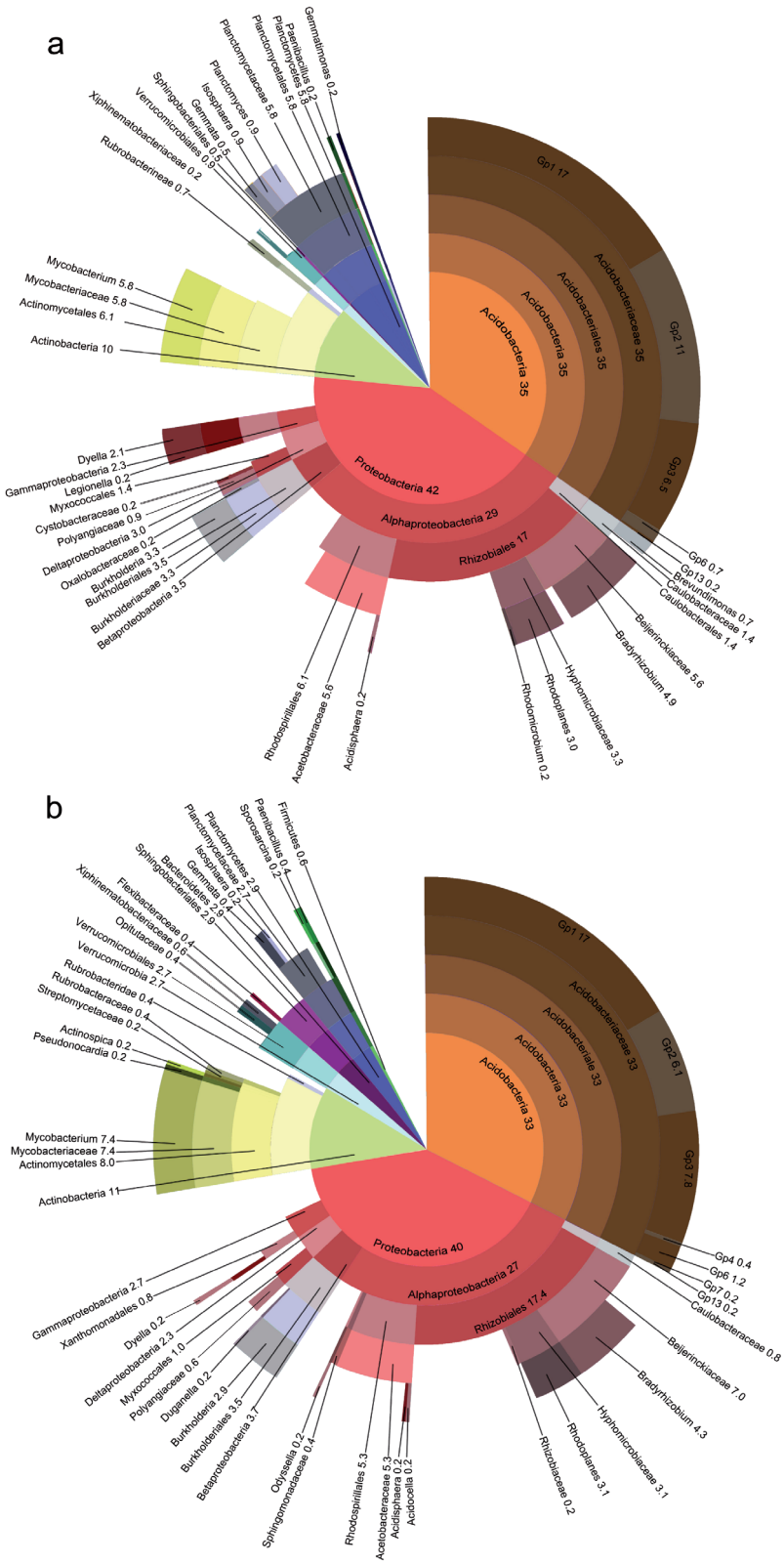

Fig. 2. Taxonomic breakdown of classified 16S rRNA gene sequences in the bacterial community of the humus layer of (**a**) control (429 sequences) and (**b**) lead-contaminated (488 sequences) areas. The taxonomic classification was performed using the Identifier from the Ribosomal Database Project (RDP) and BLAST NCBI. The central pie shows percentages by phylum, and is followed by class, order, family, and genus as the outermost annulus (blank areas represent unclassified taxa). The numbers indicate the relative frequency of taxonomic groups at each annulus expressed in percentage. The statistical significance of differences in the relative abundance of taxa between the control and polluted areas was tested using *t*-test, but no significant differences were detected.

polluted samples. The structure of the fungal community is illustrated at five taxonomic levels in pie diagrams (Fig. 3). The dominant phylum of the control area, the Basidiomycota, decreased to approximately half in the polluted area (from 62.9% to 35.9% frequency) (Fig. 3), suggesting that OTUs related to this phylum may have been suffering from the high concentrations of lead. The frequency of lower taxonomic levels also differed between control and polluted areas in this phylum. The most frequent genus of the control area, *Lactarius*, as well as other major genera *Cortinarius* and *Piloderma*, were statistically significantly reduced in frequency by lead contamination ($t = 5.33$, $p = 0.006$; $t = 3.14$, $p = 0.035$; $t = 3.54$, $p = 0.024$, respectively), suggesting that these genera suffer in the presence of lead. In contrast, the frequency of the genus *Thelephora* was higher in the polluted than control areas 14.1% *vs*. 0%; $t = -2.82$, $p = 0.048$) (Fig. 3), thereby suggesting that members of this genus have a relative selective advantage due to a higher tolerance to lead. The frequency of another major phylum, Ascomycota, almost doubled in polluted in comparison with control areas (41.3% *vs*. 22.5% frequency) (Fig. 3), implying that this group may be more tolerant to lead than Basidiomycota. Although all major taxa (frequency ≥ 5%) within the phylum Ascomycota also increased by up to 2.5 times under lead contamination (Fig. 3), no statistically significant differences could be detected between the areas, apparently due to low numbers of sequences. This, however, does indicate that there were no major changes in the Ascomycota community structure, but rather a general increase in the frequency of Ascomycota. The frequency of Chytridiomycota (on average 4.15% across the areas) was equal in the control and polluted areas and so was the frequency of higher taxonomic levels up to family in this phylum (Fig. 3).

Discussion

Bacterial diversity remained uncharged

The bacterial community observed in Hälvälä was similar to that observed in conifer forest soil by Lesaulnier *et al*. (2008), especially at the higher taxonomic levels (phylum, class and order). The bacterial community structure in both the control and polluted areas thus appeared typical for that type of environment.

In the soil, heavy metals exhibit toxic effects on soil microbes, which may decrease microbial level of activities and quantities (both biomass and diversity). For example, both the soil bacterial enzyme activity and biomass decreased as the Pb level increased from 0 to 500 mg kg^{-1} (Khan *et al*. 2010). The bacterial community shifted in 2 weeks when exposed to 5 mg kg^{-1} Cd and 500 mg kg^{-1} Pb (Khan *et al*. 2010). In the present study the bacterial community in the contaminated area was not significantly different from that in the control area. Similar results have been reported by Grandlic *et al*. (2006), who found that lead affected bacterial community structure of neither culturable nor nonculturable species. Other studies investigating effects of lead on bacterial biomass, community structure, and activity, have, however, indicated reductions in bacterial biomass and diversity (Hu *et al*. 2007), and lead was also recently found to be a strongly toxic element inhibiting microbial activities (Wang *et al*. 2010).

The reliability of the DNA approach used in our study is supported by the fact that the same DNA preparations were used as templates for determination of both bacterial and fungal diversity. The observed changes in the fungal richness, diversity and community structure in the polluted area indicate that the stability and lack of change of the bacterial community under lead contamination was not due to our method as such, being unable to detect changes in microbial community structure.

The lack of significant changes in the diversity of the bacterial community was contrary to our expectations. An explanation may be found in our earlier studies in Hälvälä. Although the total lead concentration is extremely high, the bioavailability of lead is rather low (Hui *et al*. 2009; S. Selonen unpubl. data). Even a low level of bioavailable lead should, however, have an impact on the bacteria, since already 0.35 mM lead limited the growth of a majority of cultivable bacteria from clean soil (Hui *et al*. 2009). An uneven distribution of the lead may contribute to the lack of measurable impact on the bacterial

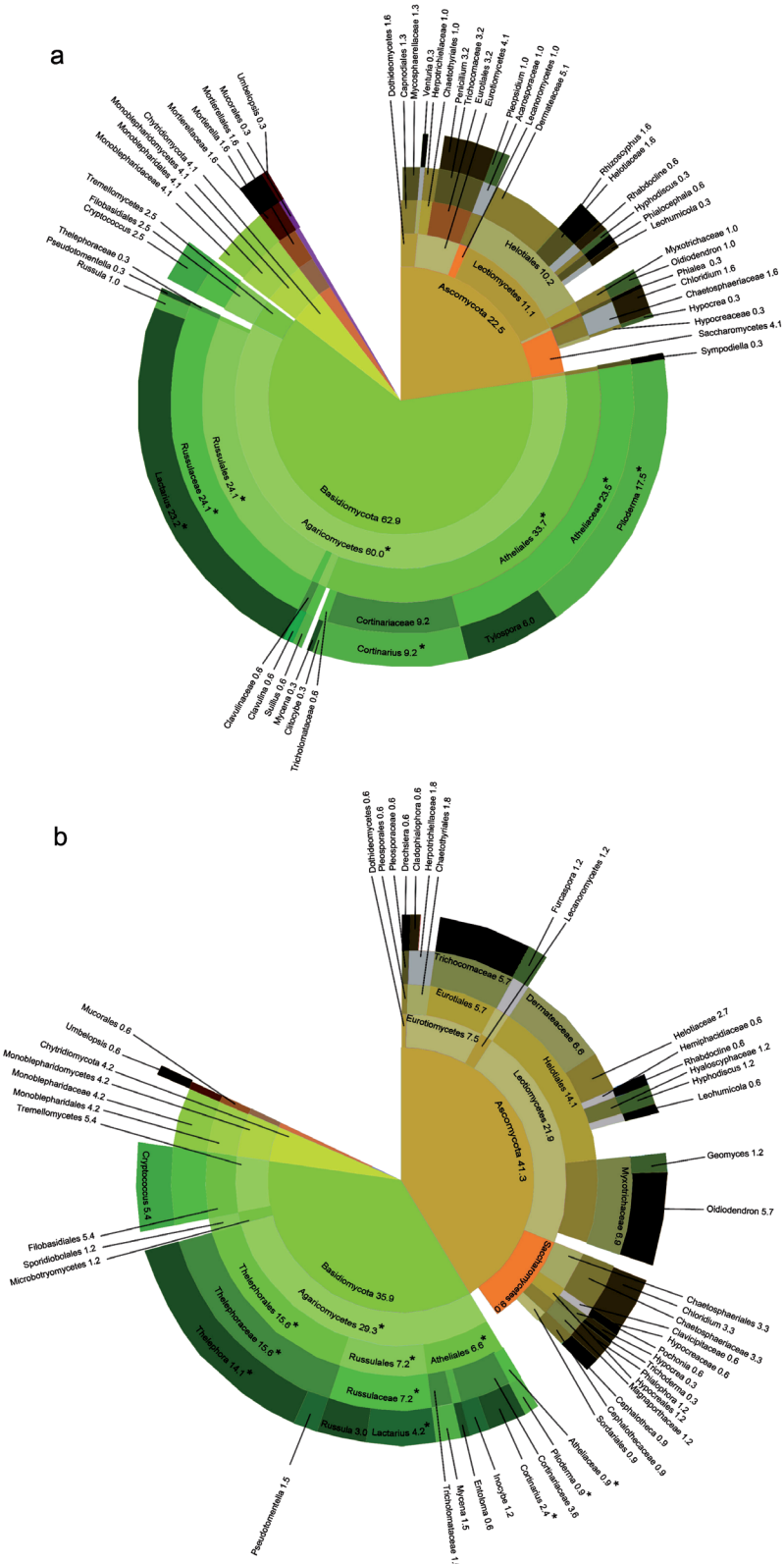

Fig. 3. Breakdown of the fungal community as classified by BLAST NCBI with fungal ITS gene in (**a**) control (315 sequences) and (**b**) lead contaminated (334 sequences) areas. The central pie shows percentages by phylum, and is followed by class, order, family, and genus (blank areas represent unclassified taxa). Numbers indicate the relative frequency of taxonomic groups at each annulus expressed in percentage. The asterisks indicate those taxa that differed in frequency between the control and contaminated areas (*t*-test: $p \leq 0.05$).

community structure. On the micro-scale such uneven distribution is difficult to assess, but on a larger scale (repeat samples) we know that large fluctuations in lead concentration do occur (Hui *et al*. 2009, Rantalainen *et al*. 2006). Finally, part of the bacterial community originally present at the formerly clean site may have evolved by slowly acquiring lead resistance traits. The shooting range has been in use for decades, and the new sector is still actively used. This has given time for bacteria to adapt to the presence of lead. Pointing to an adapted bacterial community is the recent finding that the frequency of lead resistant cultivable bacteria was elevated in the lead contaminated soil (Hui *et al*. 2009). Similar results were reported by Bååth *et al*. (2005), who showed that in a naturally lead-enriched forest soil, the bacterial community tolerance to lead had increased. Interestingly, however, although the proportion of lead resistant cultivable bacteria had increased by two orders of magnitude in the lead-contaminated Hälvälä soil, the final portion of colony-forming units growing on plates with 1.8 mM of water soluble lead was only 0.14% of the total number of colonies on lead-free plates. The lead concentration used in the plates was in the same range as the highest bioavailable level (ca. 1 mM) in the contaminated soil (Hui *et al*. 2009). The fact that lead-sensitive bacteria were present in high numbers suggests that the *in situ* bioavailability of the lead is low, as also shown by direct measurements using bioreporters (Hui *et al*. 2009). Thus, although the frequency of lead resistant bacteria increases, the majority of the bacteria remain lead sensitive, and therefore the effect of lead on the bacterial community may be too subtle to be observed by direct cloning of amplified rRNA gene fragments using the isolated total DNA as a template. The results presented here and earlier (Hui *et al*. 2009) suggest that a main strategy for bacteria to cope with high lead concentrations is to colonize micro-environments where lead does not form a selective pressure.

Fungal diversity decreased

In the Hälvälä area, both fungal and bacterial biomass have earlier been reported to be negatively affected by lead (Rantalainen *et al*. 2006), and the fungal biomass has been found to decrease more than the bacterial biomass in the lead contaminated areas (S. Selonen unpubl. data), suggesting that the fungal community is more influenced by lead than the bacterial community. In line with this, we observed a reduction in the predicted total fungal richness (S_{Chao1}) due to lead contamination. This observation contradicts findings by Khan *et al*. (2010), who reported that in a short-term exposure to lead, the bacteria were more sensitive than fungi. A possible explanation for this discrepancy is that the fungi react more slowly than bacteria, which is supported by the finding that the amount of fungi decreases as a function of incubation time (Wang *et al*. 2010). Furthermore, exposing a previously uncontaminated area to lead is likely to have a dramatic initial effect on the microbiota, after which the bacterial and fungal strains either begin to adapt and acquire resistance traits, or the initially sensitive microbial community is replaced by lead resistant microbes.

Responses of fungi to lead contamination varied among genera

Of the observed fungi, *Thelephora terrestris* was earlier shown to exhibit tolerance to high lead concentrations in liquid cultures, although the author in this case suspected precipitation of lead in the liquid media as a reason (Tam 1995). However, also McCreight and Schroeder (1982) reported growth of *T. terrestris* being arrested on agar media only at lead concentration of 200 μg ml^{-1}. Our previous study (Hui *et al*. 2011) confirms this observation. The genus *Thelephora* was elevated under Pb contamination based on the 454 pyrosequencing data for "in-growth bags". The genus *Thelephora* was relatively abundant in the polluted area, while the whole family Theleforaceae represented a very small portion of the total fungal diversity in the control area.

The genus *Cortinarius* has a relatively high diversity in boreal forest soil (Niskanen *et al*. 2008). In our study, five *Cortinarius* OTUs were spotted in the control area while two were observed in the polluted area, which suggests a

variation in lead tolerance among *Cortinarius* species. The higher abundance of *Cortinarius* in the control than in the polluted areas may reflect lead sensitivity of some, but not all *Cortinarius* species. Alternatively, lead sensitive fungi may be able to colonize lead free micro-environments in the polluted areas, as suggested for bacteria (Hui *et al*. 2009).

The genus *Lactarius* was mainly composed of *L. rufus* that has been reported as a common species in boreal coniferous forest soils (Perkiömäki and Fritze 2005, Moilanen *et al*. 2006). Krupa and Kozdrój (2007) suggested that the ectomycorrhizal *L. rufus* is able to assist its host plant (pine seedling) by restricting translocation of Zn(II), Cd(II) or Pb(II) from roots to shoots in a heavy metal polluted area, indicating that this fungal species is one of the survivors under such contaminated circumstances. In our study, *L. rufus* was observed in the polluted area, but at lower frequencies than in the control area, indicating this species to have either a partial lead resistance, or a capacity to colonize lead free micro-environments, as proposed for *Cortinarius*. As *Lactarius*, *Piloderma* is a common ectomycorrhizal fungal genus that colonizes pine roots (DeBellis *et al*. 2006), but the effect of lead on this genus is poorly known.

Responses to lead were different for bacteria and fungi

The apparent difference between fungi and bacteria in their strategy to avoid lead toxicity may have to do with the differences in mode of growth and morphology of these two kingdoms. Bacteria are single cell organisms that are directly in contact and thus directly affected by only the local micro-scale environment, while fungi grow as hyphae, some section of which is likely to be exposed to lead while growing in contaminated forest soil. This type of local exposure influences even primitive multi-cellular organisms more broadly than single cell organisms.

In the case of the bacteria, we did not observe significant replacement of typically lead sensitive genera or phyla with lead resistant ones, which suggests that indigenous bacteria in the humus do indeed adapt and evolve rather than being replaced. In contrast, the clear, although not very extensive, change observed in the fungal community structure suggests that in the case of fungi, replacement rather than adaptation is the strategy used to cope with lead in the environment. This may be related to the well documented phenomenon of rapid adaptation of bacterial strains and communities through horizontal gene transfer (Björklöf *et al*. 1995, Sarand *et al*. 2000, Syvänen 1985), whereas in fungi such strategy is slow and rare (Khaldi *et al*. 2008, Marcet-Houben and Gabaldon 2010), if at all available.

Conclusions

The ecological resilience observed in earlier studies — absence of visual differences in the lead contaminated forest compared to the control, and a lack of change in the flora — was confirmed in the case of bacterial communities. No effect of lead contamination was found on bacterial richness and diversity, while fungal richness and diversity were significantly different between lead contaminated and control areas. Some OTUs assigned to *Basidiomycota* were clearly affected and were much rarer in the lead contaminated areas. However, even in the case of fungi, genera that were deemed sensitive were not totally absent from the contaminated area — only their relative frequency was significantly reduced.

The observed difference between bacteria and fungi in their response to lead suggests a difference in their strategy to avoid the contaminant. Bacteria may be more capable of avoiding lead in the microenvironments of boreal forest soil than fungi. Alternatively, bacterial species adapt more readily by acquiring new traits, thereby avoiding being replaced by new species. The analyses performed here do not, however, allow for definite conclusions regarding these mechanisms.

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